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The Lamprey: A jawless vertebrate model system for examining origin of the neural crest and other vertebrate traits

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Summary

Lampreys are a group of jawless fishes that serve as an important point of comparison for studies of vertebrate evolution. Lampreys and hagfishes are agnathan fishes, the cyclostomes, which sit at a crucial phylogenetic position as the only living sister group of the jawed vertebrates. Comparisons between cyclostomes and jawed vertebrates can help identify shared derived (i.e. synapomorphic) traits that might have been inherited from ancestral early vertebrates, if unlikely to have arisen convergently by chance. One example of a uniquely vertebrate trait is the neural crest, an embryonic tissue that produces many cell types crucial to vertebrate features, such as the craniofacial skeleton, pigmentation of the skin, and much of the peripheral nervous system (Gans and Northcutt, 1983). Invertebrate chordates arguably lack unambiguous neural crest homologs, yet have cells with some similarities, making comparisons with lampreys and jawed vertebrates essential for inferring characteristics of development in early vertebrates, and how they may have evolved from nonvertebrate chordates. Here we review recent research on cyclostome neural crest development, including research on lamprey gene regulatory networks and differentiated neural crest fates.

Keywords

Neural crest; lamprey; neural crest derivatives; vertebrate evolution

Introduction[1]

Lampreys are jawless fishes (or agnathans), closely related to other living vertebrates, the jawed vertebrates (or gnathostomes). They, along with hagfish, are the only known surviving lineage of once diverse groups of jawless fishes. Living cyclostomes are modern yet they have some anatomic elements that appear to be retained from primitive members of their own groups, and possibly of primitive ancestral vertebrates. Lampreys are readily

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[1]Abbreviations. Mya: million years ago. NC: neural crest.

obtainable, and comparisons between lampreys and vertebrates are useful for the identification of developmental traits that are putatively derived from ancestral vertebrates. Of the two cyclostome groups, lampreys are the more experimentally tractable developmental models, and work has been done on a variety of species. As has been discussed recently (Gans and Northcutt, 1983; Shimeld and Donoghue, 2012), lamprey embryos are amenable to *in situ* hybridization, antibody staining, microinjection, morpholino oligonucleotide injection, and chemical inhibitor treatment (for protocols see: Nikitina et al., 2009a; Nikitina et al., 2009b; Nikitina et al., 2009c; Sauka-Spengler, 2009). Additionally, the lamprey *Petromyzon marinus* has been the focus of a recent genome project (Smith et al., 2013). The genome of this species (and lampreys in general; see Kuraku and Kuratani, 2006) is difficult to fully assemble in part because of high percentage GC content, in contrast to the more typical GC content of the lamprey mitochondrial genome (Lee and Kocher, 1995), but this resource will be an essential aspect of future lamprey studies. There are additional germline bacterial artificial chromosome resources for *P. marinus* and *Lethenteron japonicum* (Smith et al., 2010; Mehta et al. 2013), which is important because lampreys undergo genomic reduction during their maturation (Mallatt, 1981; Rovainen and Schieber, 1975; Smith et al., 2009).

Lampreys are an increasingly useful model organism and have been used successfully by a growing field of researchers (McCauley and Kuratani, 2008; Shimeld and Donoghue, 2012). Lamprey embryology and developmental genetics have already been crucial in addressing a number of key aspects of early vertebrate evolution, including the evolution of the jaw and evolution of the adaptive immune system (Kuratani et al., 2002; Langille and Hall, 1988a; Shigetani et al., 2002; Shimeld and Donoghue, 2012). Other recent reviews have focused on the developmental biology of lampreys, and their use in a broad variety of experimental contexts (Martin et al., 2009; Shimeld and Donoghue, 2012). In this review, we particularly focus on the ways in which lamprey data have facilitated understanding of the evolution of neural crest.

I. Lamprey phylogeny, anatomy, and fossil record

Lampreys and hagfish are jawless fishes with anatomic characters that unambiguously suggest a relatively close relationship with jawless vertebrates. The precise phylogenetic position of hagfish and lampreys relative to the jawed vertebrates has been a difficult issue to resolve, with many early phylogenetic analyses of morphological datasets suggesting that lampreys and jawed vertebrates are sister groups (to the exclusion of hagfish), but molecular phylogenetic data have supported a close relationship between hagfish and lampreys, suggesting they comprise a monophyletic group (Kuraku et al., 1999; Langille and Hall, 1988a). This has remained a contentious issue, but analyses of microRNA sequences, reexamination of morphological datasets, and recent morphological analyses of hagfish (De Beer, 1937; Heimberg et al., 2010; Janvier, 2010; Oisi et al., 2013) suggest that hagfish and lampreys are sister groups, comprising a group called the cyclostomes (See Fig. 1). Of the two cyclostome groups, hagfish are more difficult to acquire, but have recently begun receiving significant attention (Gess et al., 2006; Janvier, 2007; Ota and Kuratani, 2007). Lampreys are by far more accessible, and have been the subject of more developmental analyses. The phylogenetic position of lampreys makes them particularly useful for

comparisons with jawed vertebrates, as traits held in common between these groups are possibly homologous by descent.

Lampreys are divided into three major taxa, with a single monophyletic group in the northern hemisphere (including well-known genera such as *Petromyzon*, *Ichthyomyzon*, *Lethenteron*, and *Lampetra*), and two groups (the Geotria and Mordacia) in the southern hemisphere (Gess et al., 2006; Gill et al., 2003). Size of adult lampreys varies considerably, and partially depends on life history. Some lampreys feed only as larvae, while others parasitize on blood or flesh as adults; parasitic lampreys achieve a larger body size.

Lampreys have quite distinct anatomies at larval (ammocoete) and adult stages. Larval lampreys are diminutive fish that burrow in silty river bottoms, filtering microscopic food particles from the passing current. Their skin is smooth, they lack fully developed eyes, and their mouth opens anteriorly into a space covered by an enclosure projected from the snout, the oral hood. Ammocoetes have a specialized muscular structure, the velum, which forms from the mandibular arch (Gess et al., 2006; Hardisty and Rovainen, 1982). Rhythmic velar contractions ensure there is sufficient water flow through the pharynx to support both feeding and respiration (Bardack and Zangerl, 1968; Mallatt, 1981; Rovainen and Schieber, 1975).

Ammocoetes possess a cartilaginous skeleton that can be described as being divided into viscerocranial and neurocranial regions (See Fig. 2F). Cartilaginous elements arising from the pharyngeal arches fuse to form the branchial basket, which largely comprises the lamprey viscerocranium (Langille and Hall, 1988a; Lund and Janvier, 1986). The branchial basket appears to provide elastic recoil to counteract the movements of pharyngeal muscles (Bardack and Richardson, 1977; Martin et al., 2009). A second major skeletal structure, the mucocartilage, is a single fused connective tissue that reinforces the oral hood and oral apparatus. The mucocartilage has a unique composition, and is only found in ammocoetes. The ammocoete neurocranium includes two parachordal elements and an anterior parachordal, together referred to as trabeculae (Janvier, 2007; Langille and Hall, 1988a). Additional skeletal elements include a pericardial capsule, and otic and nasal capsules (De Beer, 1937; Janvier, 1996), which have received little attention from developmental geneticists.

Larval lampreys retain the ammocoete body plan for at least several years and, after reaching a suitable size, they go through a significant metamorphosis during which many tissues are reformed and rearranged into adult body structures (De Beer, 1937). These transformations include final development of eye structures, the degradation of mucocartilaginous skeletal elements and their replacement with cartilaginous elements of the adult, and the alteration of the velum from a flow-generating structure to one that acts as a valve to separate feeding (i.e. esophageal) and respiratory (i.e. pharyngeal) channels (Hardisty, 1979). Cartilaginous elements that replace the mucocartilage include an annular cartilage that reinforces the rostral oral opening, paired styliform cartilages, dorsal and lateral elements, cartilaginous elements on their rasping tongue, and a piston cartilage, a key skeletal component that generates the force necessary to feed (De Beer, 1937; Johnels, 1948).

As adults, lampreys are elongated, eel-like fish. As in the ammocoete, the adult integument is smooth, without scales or ossified structures (Hardisty and Potter, 1971). The lamprey mouth, instead of having an opposable jaw, opens as a round sucker with keratinized 'teeth'. Their fully developed eyes lack intrinsic musculature, and they have a pineal eye that sits on the cranial dorsal midline (Hardisty and Potter, 1971). Their pharynx is perforated by seven round gill slits, which open into muscular pharyngeal pouches. They lack paired fins, but have dorsal, caudal, and anal fins, and some species are quite physically powerful. Many lamprey species are parasitic as adults, and they attach to fish, rasp through the fish integument, and feed on blood or flesh (Hardisty and Potter, 1971). While adults are attached via the sucker, they alternately contract left or right pharyngeal pouches to draw oxygenated water into their pharynx for respiration (Hardisty, 1979). Additional pore musculature controls the aperture of the gill slits.

Lampreys have a lengthy fossil record, and are sometimes regarded as 'living fossils' because of their strong resemblance to early fossil material (Gess et al., 2006; Hardisty, 1979; Janvier, 2007). Careful examination of these fossils might provide clues as to which morphological traits have been most stable in lampreys. The earliest fossil lamprey is *Priscomyzon*, which dates from 360 million years ago (Mya) (Chang et al., 2006; Gess et al., 2006). *Priscomyzon* shows multiple anatomic traits found in modern lamprey, including a round mouth, a piston cartilage, and seven pharyngeal pouches (Chang et al., 2006; Gess et al., 2006). These traits, and in particular the piston cartilage, are consistent with the animal having had a predatory adult stage, despite its very small size of 4 cm (Gess et al., 2006; Janvier, 2006). Other fossil lampreys include *Mayomyzon* (Bardack and Zangerl, 1968; Janvier, 2007), 1968), *Hardistiella* (Janvier, 2007; Lund and Janvier, 1986), and *Pipiscius* (Bardack and Richardson, 1977; Janvier, 1996), all from the late carboniferous (305 Mya; Janvier, 2007; Janvier et al., 2006). These fossils are also of very small size, and the juxtaposition of small size and morphologies found in adult lampreys might suggest that *Mayomyzon* lacked a lengthy morphologically distinct larval stage (Gans and Northcutt, 1983; Glenn Northcutt, 2005; Janvier, 1996; Northcutt and Gans, 1983). However, the rostral tip of *Mayomyzon* shows a smaller oral hood than modern lampreys, leading to the suggestion that *Mayomyzon* might have been nonparasitic (Hardisty, 1979). Another lamprey from 125 Mya, *Mesomyzon mengae* (Chang et al., 2006; Piavis, 1971; Richardson and Wright, 2003) exhibits a closer resemblance to living lampreys, with a slightly larger body size and a lengthened snout. All lamprey fossils have skin without a dermal skeleton, a trait likely to have arisen in stem gnathostomes (Gess et al., 2006; Janvier, 2006; Tahara, 1988). These lamprey fossils date to 360 Mya, while the minimum age of hagfish is about 300 Mya (Janvier, 2007; Yamazaki et al., 2003). Assuming monophyletic cyclostomes, based on the ages of stem gnathostome fossils, it is likely that cyclostomes and gnathostomes split no less than 475–500 Mya (Janvier, 2007; Tahara, 1988).

These fossils confirm that some aspects of lamprey development date from early in their evolution and that some traits, like the presence of a piston cartilage, show apparent stability over time. Other traits appear to be quite different in early lampreys. Notably all the early lamprey fossils are small in size compared to current lampreys, with no fossil yet discovered being longer than 10 cm, and each of these fossils has traits, like a piston cartilage, that is

associated with the adult morphology of living lampreys (Janvier, 2007). This suggests that early adult lampreys might have been quite small, consistent with a possibly abbreviated or absent larval stage (Janvier, 1996; Koltzoff, 1901). This raises the possibility that traits associated with larval lampreys (including mucocartilage, delayed ocular development, and distinct velar morphology) might have been secondary modifications of the lamprey form. Fossils of juvenile lampreys will be necessary to be certain, but anterior-most larval structures might be particularly derived in lampreys as a result of the evolution of larval feeding strategies.

Another interesting use of fossils for developmental geneticists is that they indicate some aspects of development that are particularly likely to be representative of primitive conditions. One such example is the muscularized pharyngeal pouch of lampreys. Among living animals, these structures are unique to lampreys, and were once considered an oddity of lamprey anatomy. The discovery of a stem-gnathostome fossil (i.e. an ancient jawless fish more closely related to jawed vertebrates than to cyclostomes) of *Endeiolepis* (370 Mya; Janvier et al., 2006) with very similar pharyngeal pouches suggests that pharyngeal pouches were a general trait of primitive vertebrates that have been secondarily lost in living gnathostomes. Thus, it is possible that lamprey pharyngeal pouch muscle plates represent an example of a primitive condition once common among early vertebrates. If so, then study of neural crest and muscle development in pharyngeal pouch structures offers potentially unique insights into the evolution of a muscularized pharynx, thought to be a key element of the transition from invertebrate filterfeeding to vertebrate predation (Gans and Northcutt, 1983; Glenn Northcutt, 2005; Horigome et al., 1999; Northcutt and Gans, 1983).

II. Lamprey neural crest embryology and morphology

Early embryology of lampreys has been described in several species, including the European brook lamprey *Lampetra fluviatilis* (Damas, 1944; Horigome et al., 1999), the Atlantic sea lamprey *P. marinus* (Horigome et al., 1999; Piavis, 1971; Richardson and Wright, 2003), *Lampetra reissneri* (Horigome et al., 1999; McCauley and Bronner-Fraser, 2003; Tahara, 1988), and the Pacific lamprey *Entosphenus tridentatus* (McCauley and Bronner-Fraser, 2003; Yamazaki et al., 2003). Generally, embryology of these lamprey species is quite similar, varying only in developmental rate. Rather than relying on staging by embryonic day, which varies between species, lamprey research commonly follows the staging table of (McCauley and Bronner-Fraser, 2003; Tahara, 1988). Lamprey embryos are yolky and quite large (See Fig. 2). Early cleavage is radial and holoblastic, with the mesolecithal yolk distribution leading to significant differences in size of animal and vegetal blastomeres. Following gastrulation, lateral edges of the neural plate rise and fuse at the midline, producing a neural rod that includes precursors of neural crest. The neural lumen is produced secondarily by cavitation.

In vertebrates, including lamprey, the neural crest is a transient multipotential and stem cell-like population that produces a wide variety of cell types important to the vertebrate body plans, including skeletal, glial, and pigment cell types. In jawed vertebrates, neural crest cells originate from cells between the neural and non-neural ectoderm, in a region called the neural plate border. Cells from this region elevate during neural tube formation, and

generally come to lie in the dorsal neural tube. Neural crest cells delaminate from the adjoining neurepithelium and ectoderm, go through an epithelial to mesenchymal transition, and migrate away from the neural tube. Neural crest cells migrate via routes that vary by axial level and also by species. Generally, they migrate either ventromedially, through or around the somites, or they travel through the dorsolateral pathway, remaining subjacent to epidermis, while migrating to other locations (See Fig. 3). Cells may also remain dorsally and give rise to structures of median and dorsal fins.

Lamprey neural crest cells were first identified by Koltzoff (Koltzoff, 1901; McCauley and Bronner-Fraser, 2003). For a discussion of early morphological studies and an important discussion of *L. fluviatilis* embryonic cranial morphology, please refer to Damas (1944). More recently, Horigome et al (1999) and Kuratani (1997) examined the morphology of *L. japonica* neural crest cells at premigratory, migratory, and postmigratory stages by a combination of electron microscopy and DiI labeling. *L. japonica* embryology is very similar to that of *P. marinus*, and these data offer an excellent model of lamprey neural crest development. Following formation of the lamprey neural rod, presumably, neural crest precursors sit at the dorsal neural tube. In *L. japonica*, cranial neural crest cells are visible as bulges from the dorsal neural tube at about Tahara St. 20 (Horigome et al., 1999; Meulemans and Bronner-Fraser, 2004). Shortly thereafter, at about Tahara state 20.5, cells begin to delaminate from the neural tube, and they migrate in three streams, termed the trigeminal, hyoid, and branchial streams (Horigome et al., 1999; Meulemans and Bronner-Fraser, 2004). Trigeminal crest originates from midbrain levels to the second rhombomere, and cells migrate via the dorsolateral pathway over the forebrain and mandibular mesoderm. Hyoid stream neural crest cells migrate ventrally from a position adjacent to rhombomere 4, beneath the otic primordium, and into a superficial position within the hyoid arch, bounded by first and second pharyngeal pouches. Branchial crest cells initiate migration from rhombomere 6 and more posterior positions. The precise posterior border of branchial (posteriormost cranial) neural crest cells is indistinct, and migration of trunk neural crest has received little attention in lampreys. Little is known about presumed migration into cardiac and enteric positions. Lamprey neural crest has been shown to migrate along dorsolateral migration pathways in *L. japonica* and *P. marinus* (Horigome et al., 1999; McCauley and Bronner-Fraser, 2003; Meulemans and Bronner-Fraser, 2004), but fate-mapping data show that cranial neural crest cells also migrate along a ventromedial pathway in *P. marinus* (Light et al., 2005; McCauley and Bronner-Fraser, 2003; Sauka-Spengler and Bronner-Fraser, 2006). Comparable fate-mapping analyses have not been completed in *L. japonica*, but we presume that lamprey cranial crest cells are likely to use both pathways. In gnathostomes, cells traveling the dorsolateral pathway predominantly differentiate into pigment cells, but it is not clear whether particular neural crest fates are associated with, or restricted to, a particular pathway in lampreys.

McCauley and Bronner-Fraser (2003) showed that late-emigrating labeled neural crest cells are capable of migrating to both dorsal and ventral positions within pharyngeal arches. A difference between gnathostome and lamprey neural crest migration is that lamprey cranial neural crest cells migrating into the pharyngeal region posterior to the hyoid arch appear relatively unconstrained, and continue migrating anteriorly and posteriorly until the

formation of the posterior pharyngeal (i.e. branchial) arches (McCauley and Bronner-Fraser, 2003).

As in *Xenopus* (Krotoski and Bronner-Fraser, 1986), some lamprey neural crest migrates adjacent to the notochord: lamprey melanophores are visible surrounding the notochord (Kuratani et al., 1997). It is likely that these cells migrate from the ventral pathway.

III. Gene regulatory networks active in early neural crest cell induction, specification, and maintenance

Extensive examination of neural crest formation in a variety of model species led to the contention that neural crest cell development arises through the activity of a gene regulatory network that is largely conserved throughout vertebrates (Meulemans and Bronner-Fraser, 2004). Interactions between neural crest regulatory network genes result in successive refinement of the distinct fate and behavior of neural crest, establishment of cellular conditions for the maintenance of neural crest fate, establishment of receptive ability to environmental cues governing further differentiation, and control of the epithelial to mesenchymal transformation that crest undergoes in order to migrate away from the neural tube. This network is induced when information from BMP, Wnt, and FGF signaling pathways progressively subdivides ectoderm into three regions: the neural plate, the non-neural ectoderm, and the intervening neural plate border region. Proteins acting as ‘neural plate border specifiers’ include Zic, Msx, Dlx3/5, and Pax3/7 (Meulemans and Bronner-Fraser, 2004). Border specifiers, along with other inductive signals, are responsible for activating ‘neural crest specifiers’, a set of genes whose combined, overlapping expression pattern is indicative of presumptive neural crest cells. The neural crest specification genes include *Sox9, Sox10, Msx1/2, AP2, c-Myc, Snail*, and *Slug*. Additional genes generally expressed in early neural crest include *Id* and *Twist* homologs (Meulemans and Bronner-Fraser, 2004). *Xenopus* Id3 is necessary for neural crest stem cell specification and maintenance (Kee, 2005; Light et al., 2005; Sauka-Spengler and Bronner-Fraser, 2006). Neural crest specifier genes in turn activate additional effector genes responsible for activating individual functions of neural crest subtypes (Bronner-Fraser and Sauka-Spengler, 2010). Early neural crest specifier activity leads to the activation of other key effectors of neural crest fate, including altered Cadherin and RhoGTPase activity. For comprehensive reviews of early neural crest cell development and the epithelial to mesenchymal transition, please see (Bronner-Fraser and Sauka-Spengler, 2010; Kerosuo and Bronner-Fraser, 2012; Prasad et al., 2012).

Lamprey neural crest gene regulatory network—Lampreys have a crucial phylogenetic position for making inferences about the state of the neural crest gene regulatory network in early vertebrates. Careful examination of the expression patterns of fifty *P. marinus* candidate genes with roles in neural crest of gnathostomes has suggested that the lamprey *P. marinus* uses a neural crest gene regulatory network that is broadly similar to those of jawed vertebrates (Sauka-Spengler et al., 2007). The lamprey *P. marinus* embryo shows expression of *Pax3/7, MsxA*, and *Zic* (though not *DlxB*), during neural crest border specification, as well as expression of neural crest specification genes at neural crest border specification and neural crest specification stages. Subsequent analyses (Nikitina et

al., 2008) have refined this by showing that *AP2* and *MsxA* initially act upstream of other genes (*ZicA, Pax3/7, Id*, and *n-Myc*) active in the neural plate border region. These data suggest that formation of the neural crest in lampreys is broadly similar to that of other vertebrates, though there may be some differences in the timing of deployment between cyclostomes and gnathostomes. Although *Twist* and *Ets1* function as neural crest specifier genes in gnathostomes, *in situ* hybridization failed to detect expression of their homologs in premigratory or early migratory neural crest of lamprey. Rather, these genes were activated in late migrating crest cells within the branchial arches. Such differences may in part explain the changes in neural crest formation in jawed versus jawless vertebrates.

These results show that the majority of the gene regulatory network leading to neural crest formation is conserved between jawless and jawed vertebrates and was already present in the ancestors of all craniate animals. This implies that the earliest origins of neural crest took place in non-vertebrate chordates. It can be difficult to unambiguously identify a crest homolog in the latter, because their body plans are quite different, but knowledge of gene regulatory network structure can provide a supplemental means to test hypotheses about neural crest homologs.

Neural crest origins and putative neural crest homologs in the invertebrate chordates—Neural crest cell derivative fates can be broadly grouped into ectomesenchymal and neuroglial fates (Donoghue et al., 2008). The earliest origins of neural crest are unclear, but it is likely that ectomesenchymal fates of neural crest emerged in early vertebrates (Baker, 2008; Ivashkin and Adameyko, 2013). This is corroborated by the appearance of skeletal elements in fossil lampreys and in the fossils of other early vertebrates. It is possible that the earliest neural crest was a neuroepithelial lineage, and that such a lineage might be present in invertebrate chordates. Several hypotheses have suggested that neural crest originated from a single lineage of fairly differentiated cells arising from the neural border region, for instance cells similar to Rohon-Beard cells (Fritzsch and Northcutt, 1993), or from ascidian pigment cells. Another possibility is that neural crest arose from a multipotent pigment precursor cell (Abitua et al., 2012; Ivashkin and Adameyko, 2013).

Urochordates—Because all vertebrates possess neural crest cells, many have looked to the relatively closely related invertebrate chordates for clues as to the earliest origins of the neural crest. The closest relatives to vertebrates are the urochordates, including ascidians; urochordates and vertebrates are sister groups comprising the group Olfactores (Abitua et al., 2012; Delsuc et al., 2006). There have been several distinct claims about homologous tissues in ascidians. One (Jeffery et al., 2004) suggested that the A7.6 lineage, which produces migratory pigment cells, might be homologous to the neural crest. These cells are mesodermal or mesendodermal (Abitua et al., 2012; Baker, 2008; Jeffery et al., 2004), and expression analyses of their derivatives suggest the cells do not arise from the neural plate border (Jeffery et al., 2008).

Abitua et al (2012) have instead suggested that the a9.49 cell lineage is homologous to neural crest. Crucially, this lineage arises from a neural plate border region that expresses multiple neural plate border and neural crest specification genes, including homologs of

Msx, *Pax3/7*, *Zic*, *AP-2*, *ID*, and *Snail* (Abitua et al., 2012). The cells normally undergo a short but complex migration (Baker, 2008) to become pigment cells, and the lineage also expresses *FoxD*, a crucial regulator of neural crest; in *Ciona* the gene is necessary for *MITF* expression and pigment formation (Abitua et al., 2012). If the a9.49 cell represents a homolog of neural crest, then it implies the presence of at least a homologous neuroglial lineage early, in the early stem Olfactores that were the common ancestors of vertebrates and ascidians. Overexpression of Twist in a9.49 cells triggers cell migration, leading to the contention that perhaps acquisition of *Twist* expression, or expression of a similar gene, in a relatively simple neural crest homolog in stem vertebrates might have capacitated gene networks, allowing neural crest elaboration into vertebrate skeletal structures (Abitua et al., 2012).

Cephalochordates—Cephalochordates have no obvious homolog of neural crest cells, but they do have a neural border region that features very similar expression of neural plate border specification genes, including *Zic*, *Msx*, and *Pax3/7* homologs. However, *AP2*, *FoxD3*, and *Id* expression hasn't been detected in the border region, though *Snail* has been detected in this site (Sauka-Spengler and Bronner-Fraser, 2006; Yu, 2010; Yu et al., 2008). The absence of neural crest in amphioxus, as well as apparent absence of any homologous tissue within other deuterostome phyla, such as hemichordates and echinoderms, has typically been interpreted as a primitive character, implying that the earliest neural crest arose in stem vertebrates or stem Olfactores, perhaps in association with novel regulatory elements associated with newly duplicated vertebrate genes (Ota and Kuratani, 2007; Yu et al., 2008).

Another hypothesis suggests that neural crest arose from a multipotent neuroepithelial precursor cell responsible for pigmentation and light reception, which suggests the amphioxus ocellus as a possible neural crest paralog (Ivashkin and Adameyko, 2013).

Patterns of gene subfunctionalization among vertebrate duplicates suggest that core mechanisms of neural crest formation arose prior to vertebrate genome duplications (Medeiros, 2013). Neural crest-like abilities are phylogenetically widespread throughout invertebrates, and a broader understanding of the developmental underpinnings of the sensory cells found in other animals will strengthen assessments of neural crest evolution (Medeiros, 2013).

IV. Neural crest derivatives in lampreys

It is clear from many studies that migrating lamprey neural crest cells give rise to many cell types typical of the neural crest of jawed vertebrates, including melanophores, chondrocytes, and presumably other connective tissues. Ablation or removal of lamprey neural crest cells reduces pigmentation (McCauley and Bronner-Fraser, 2003; Newth, 1951; Langille and Hall, 1988b, Sauka-Spengler and Bronner-Fraser, 2006), and transplanted dorsal neural tissue introduces melanophores into ectopic sites (Newth, 1956). In lampreys, neural crest cell migration into branchial arches is consistent with a role in formation of branchial arch skeletal structures (Horigome et al., 1999; McCauley and Bronner-Fraser, 2003). Langille

and Hall (1988b) showed that surgical removal of neural crest and dorsal neural tube at premigratory stages leads to reductions in branchial skeletal elements and trabeculae.

There also are important differences in neural crest derivatives within the vertebrate lineage. For example, the structure of the autonomic system, traditionally divided into sympathetic, parasympathetic, and enteric subdivisions for mammals, is very different in other vertebrates (Nilsson, 2011). The sympathetic system of many jawed vertebrates uses a paravertebral series of sympathetic chain ganglia (Nilsson, 2011). However, interconnections between sympathetic chain ganglia are absent in elasmobranch sharks (Häming et al., 2011; Nilsson, 2011; Young, 1933), and in cyclostomes there are no sympathetic chain ganglia (Fange et al., 1963; Häming et al., 2011; Nicol, 1952). Consistent with these morphological observations, attempts to examine autonomic cell markers have shown that markers homologous to those expressed in chain ganglia of vertebrates – *Phox2*, *Ash/Ascl*, and *Hand* – are not coexpressed at early embryonic stages (Häming et al., 2011).

Certain derivatives that are typical for jawed vertebrates do not appear to be present in lampreys. Notably, lampreys do not possess an easily identifiable structure homologous to sympathetic chain ganglia (Häming et al., 2011; Johnels, 1956). At cranial levels, lampreys have autonomic pathways through the vagus nerve, and possibly through the facial and glossopharyngeal nerves (Nicol, 1952; Tretjakoff, 1927). Fibers innervating the gut (and other visceral organs) throughout much of the trunk derive from spinal neurons, similar to the condition of amphioxus (Fritzsch and Northcutt, 1993).

While there are no obvious homologs of the paravertebral structures, lampreys might have cells homologous to autonomic ganglia. Lampreys have putative autonomic nerve cells adjacent to the cloaca and peripheral nerve plexuses on kidneys and gonads (Johnels, 1956). Nakao and Ishizawa (1982) examined the ultrastructure of cloacal ganglion cells, confirming that their morphology is consistent with autonomic function. This suggests that while lamprey might lack an organized placement of autonomic ganglia in a position adjacent to the spine, homologous cell types might exist. The physiological function of these neurons isn't clear, but it is likely that they promote gut motility. Understanding the origins of these cells will be important in determining whether these might represent homologs of sympathetic ganglia.

Adrenal chromaffin cells, which derive from neural crest in jawed vertebrates, are an additional neural crest-derived cell type. Lampreys have both cardiac chromaffin cells and extracardiac chromaffin cells (Paiement and McMillan, 1975). Extracardiac chromaffin cells might be homologous to cells of the gnathostome adrenal medulla (Gaskell, 1912; Paiement and McMillan, 1975). The origin of lamprey cardiac chromaffin cells is unknown.

The lamprey heart has two chambers, with components that include neural crest-derived elements in jawed vertebrates. Embryonic lamprey hearts have been reported to have multiple valves, including a sinoatrial valve, an atrioventricular valve, and outflow valves (Farrell, 2007; Lee et al., 2013; Richardson et al., 2010; Shipley). However, the outflow valves are not necessarily homologous to the semilunar valves of amniotes (Bullock et al.,

1984; Peters, 1960; Richardson et al., 2010; Schultz et al., 1956), which include neural crest-derived cells (Jain et al., 2011; Nakamura, 2006; Smith et al., 2013).

In gnathostomes, neural crest cells differentiate into pericytes and smooth muscle cells of anterior cranial vasculature (Etchevers et al., 2001). In lampreys, defects in neural crest can lead to dilation of anterior arteries (Newth, 1956), suggesting that neural crest cells are likely to contribute to cranial vasculature. A more precise study of crest interactions with vasculature has not been completed, and it is possible that these defects arise from interactions between neural crest cells and mesoderm, or a general physiological defect (Newth, 1956). Overall the lamprey hematopoietic system is reported to be somewhat similar to that of jawed vertebrates, and there are lymphocytes thought to be homologous to B cells and T cells (Guo et al., 2009; Kasamatsu et al., 2010; Rogozin et al., 2007), though their immune system uses a completely different system of receptors, called VLR receptors, to mediate interactions with foreign molecules. There is a report that lampreys might have a rudimentary thymus (Bajoghli et al., 2011), which in vertebrates includes neural-crest derived elements (Bockman and Kirby, 1984; Foster et al., 2008; Lee et al., 2013; Müller et al., 2008). However, it is unknown whether the lamprey thymoid includes neural crest-derived cells.

Other anatomic neural crest derivatives might be absent in lampreys. Notably, lamprey peripheral neurons are not myelinated, but they are covered with presumptive Schwann cells (Bullock et al., 1984; Peters, 1960; Schultz et al., 1956). These cells may be crest derivatives, but they have not been characterized by molecular genetic methods and their origins are unclear. Interestingly, the lamprey genome has multiple genes associated with myelin production, but use and expression pattern of these genes is unclear (Smith et al., 2013). As mentioned above, lamprey eyes lack intrinsic musculature, which in jawed vertebrates is present and derived from neural crest.

There are still unstudied aspects of neural crest that could be very important in early evolution of crest. Notably, there are numerous interactions between mesoderm and neural crest during the formation of vertebrate cranial muscles. Modification of sites of muscle formation may have been a primary role of early crest. These are not even very well studied in vertebrates, so these analyses must begin in well-established systems, such as avians, zebrafish, and amphibians, that are well suited to addressing these questions.

Conclusion

Species of lampreys living today are by definition modern, competitive, and ecologically successful, yet they are morphologically similar to ancestral groups some 360 millions years distant. Lampreys are at a crucial phylogenetic position, and studies of lamprey anatomy, development, and gene regulation have provided crucial insights into the evolution of neural crest within vertebrates. There are still many open avenues of research, including the evolution of cell communication, and coordination and integration of patterning events in different tissues. Together with comparisons with other non-vertebrate chordates, studies of these gene regulatory networks in lampreys might provide an understanding of the earliest origins of a germ layer. Regardless of precisely when and how neural crest emerged,

cyclostomes, and lampreys in particular, will remain crucial for making inferences about the evolutionary elaboration of later neural crest derivatives.

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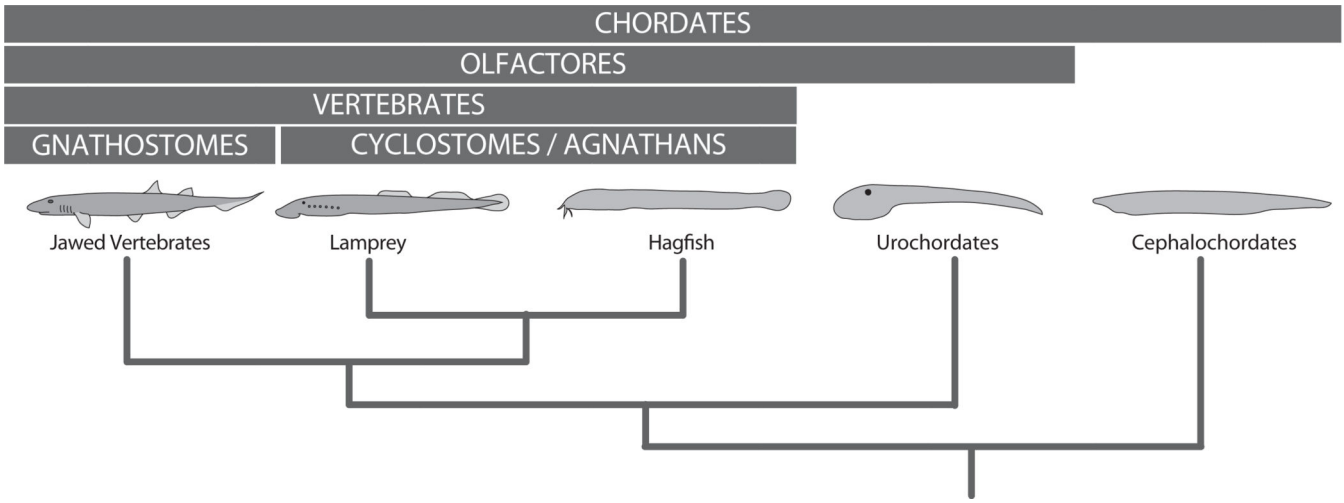

Figure 1.

Schematic cladogram of interrelationships between select chordate taxa. The labels at top indicate the names of the monophyletic groupings shown beneath.

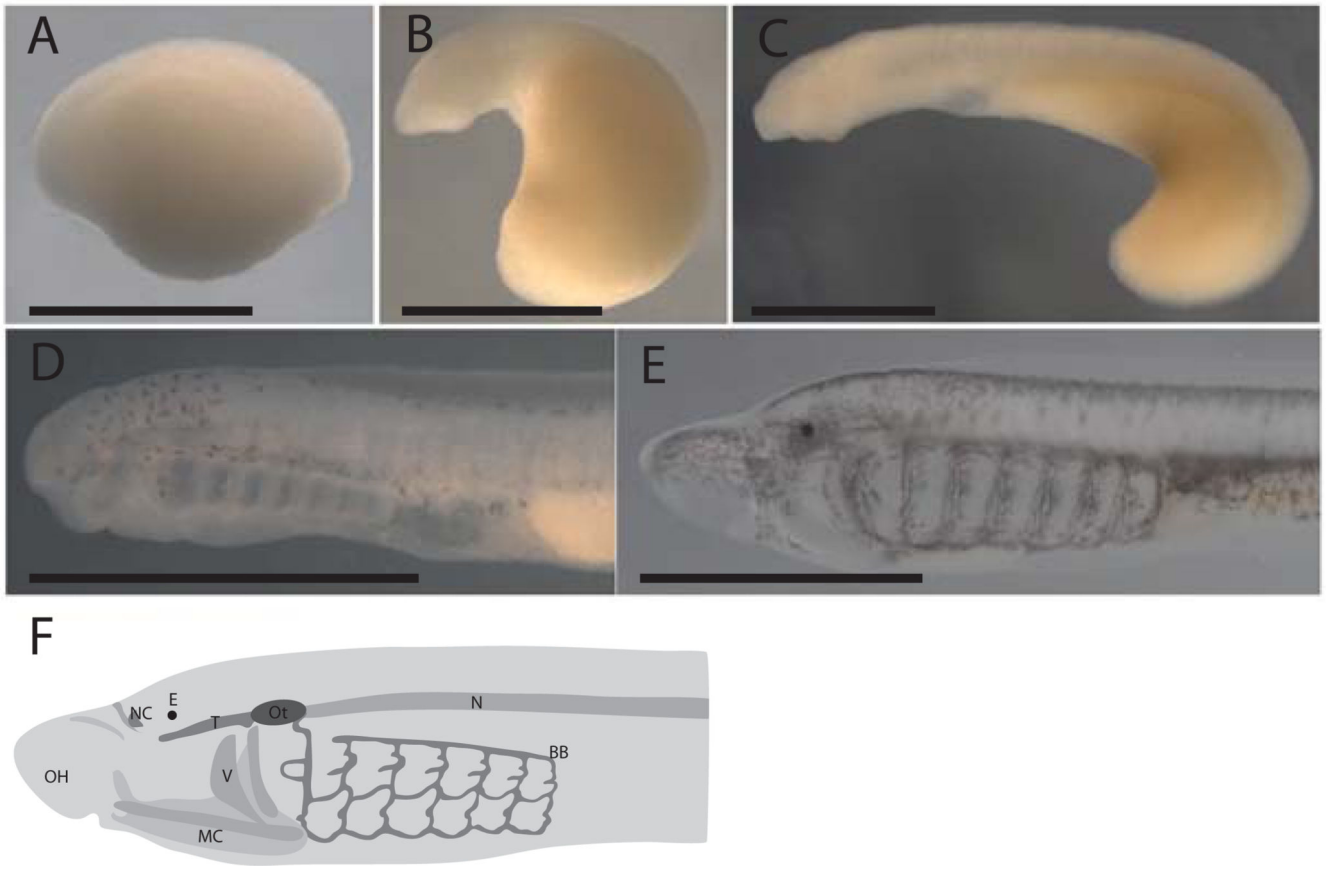

Figure 2.

External morphology during early development of the lamprey *P. marinus*. A. Embryo after neural rod formation, approximately Tahara Stage 20. B. Embryo at Tahara Stage 22. C. Embryo at Tahara 24.5. D. Embryo at T28 embryo. E. Proammocoete. F. Schematic of a young ammocoete, redrawn after De Beer (1937), and Langille and Hall (1988a). BB: branchial basket, E: eye, MC: mucocartilage, N: notochord, NC: nasal cartilage, OH: oral hood, Ot: Otic capsule, T: trabeculae, V: velum. Bar indicates 1 mm.

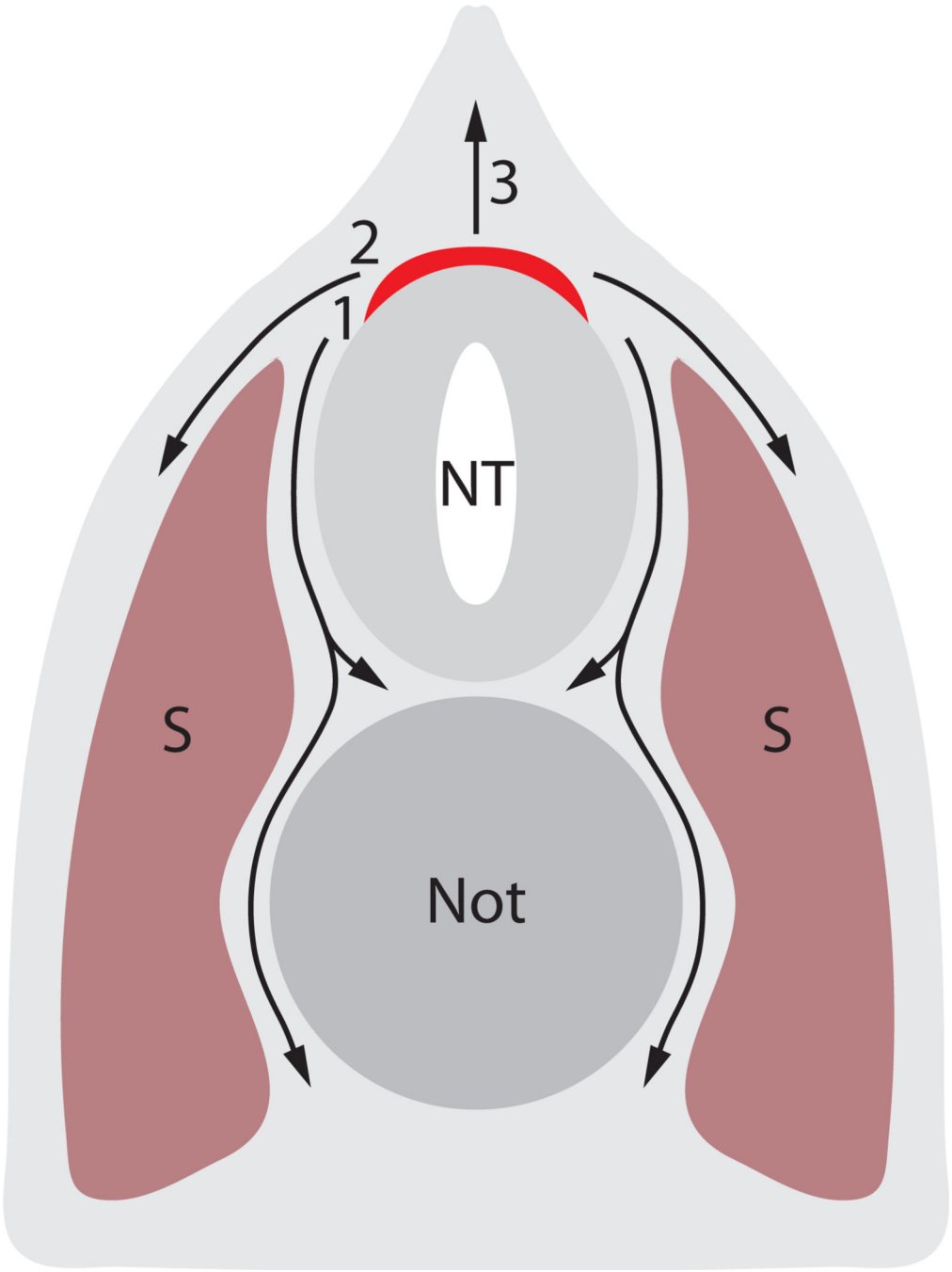

Figure 3.

Schematic diagram of neural crest migration pathways through the dorsal half of a vertebrate cross-section. Red color indicates site of origin of premigratory neural crest. 1: ventromedial migration pathway, 2: dorsolateral migration pathway, 3: dorsal migration pathway. S: somite. Not: notochord. NT: neural tube. After Krotoski and Bronner-Fraser (1986).